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## Phylogeography of competing sexual and parthenogenetic forms of a freshwater flatworm: patterns and explanations

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### Abstract

**Background:** Models of the maintenance of sex predict that one reproductive strategy, sexual or parthenogenetic, should outcompete the other. Distribution patterns may reflect the outcome of this competition as well as the effect of chance and historical events. We review the distribution data of sexual and parthenogenetic biotypes of the planarian *Schmidtea polychroa*.

**Results:** *S. polychroa* lives in allopatry or sympatry across Europe except for Central and North-Western Europe, where sexual individuals have never been reported. A phylogenetic relationship between 36 populations based on a 385 bp fragment of the mitochondrial cytochrome oxidase I gene revealed that haplotypes were often similar over large geographic distances. In North Italian lakes, however, diversity was extreme, with sequence differences of up to 5% within the same lake in both sexuals and parthenogens. Mixed populations showed "endemic" parthenogenetic lineages that presumably originated from coexisting sexuals, and distantly related ones that probably result from colonization by parthenogens independent from sexuals.

**Conclusions:** Parthenogens originated repeatedly from sexuals, mainly in Italy, but the same may apply to other Mediterranean regions (Spain, Greece). The degree of divergence between populations suggests that *S. polychroa* survived the ice ages in separate ice-free areas in Central, Eastern and Southern Europe and re-colonised Europe after the retreat of the major glaciers. Combining these results with those based on nuclear markers, the data suggest that repeated hybridisation between sexuals and parthenogenetic lineages in mixed populations maintains high levels of genetic diversity in parthenogens. This can explain why parthenogens persist in populations that were originally sexual. Exclusive parthenogenesis in central and western populations suggests better colonisation capacity, possibly because of inbreeding costs as well as hybridisation of sexuals with parthenogens.

### Background

Theory predicts that stable coexistence of sexual and parthenogenetic conspecifics must be rare [1,2]. Mixed asso-

ciations are therefore seen as a transition towards the extinction of one of the two forms [3]. A non-overlapping spatial distribution pattern of sexuals and parthenogens is

frequently found and has been termed geographic parthenogenesis [4]. Typically, the (ancestral) sexual population is located in the distribution centre of the species, while parthenogens are present at the margin of the distribution [5]. An ecological explanation for this pattern does not exist [6]. Lynch [7] proposed that parthenogens might fail to establish themselves in the presence of sexuals as backcrosses with the ancestral sexual forms may hamper the independence of purely parthenogenetic forms. But parthenogens may have better colonizing capacities: They have a higher intrinsic growth rate and do not pay the deleterious effects of population bottlenecks (e.g. inbreeding) that may act on sexual populations [7]. As a result, they may colonize areas where sexuals have difficulties establishing a population [6].

Non-adaptive explanations for geographic parthenogenesis take historical events into account, e.g. when colonisation was recent. In Europe, as elsewhere, the climate changed dramatically during the past 20,000 years from the last glacial height to the present interglacial period [8,9]. As a result, previously glaciated areas have become available for species that could not withstand the earlier low temperatures [10]. Recolonization, however, also requires migration routes that allow dispersal from 'refugia'. Hence, distributions will be affected by dispersal barriers such as mountain ranges like the Pyrenees, the Alps and the Balkans [11,10]. Using mitochondrial DNA sequences it has become possible to reconstruct the phylogenetic relationships between populations of the same species, thereby identifying the source populations of the lineages that (re)colonized Central and Northern Europe [12].

Intraspecific phylogenies may also reveal age and origin of parthenogenetic lineages [13–15]. Since mtDNA is maternally inherited, it is also a suitable tool to identify the maternal ancestors of hybrid parthenogens [16–20]. Both allow identification of the rate at which new parthenogenetic lineages arise, and to what extent the presence of sexual conspecifics is required.

*Schmidtea polychroa* is a freshwater planarian with a Europe-wide distribution. It is a simultaneous hermaphrodite with sexual and parthenogenetic biotypes and has been used for various studies on the costs and benefits of sex [21–24]. In much of Western and Central Europe sexuals are absent [25], whereas mixed populations of sexuals and parthenogens occur in Italy [25,26] and Sweden [27]. It is not known whether coexistence is in equilibrium or whether invasion and displacement are still in progress. Allozyme markers indicated a polyphyletic origin of parthenogens from sexuals [26]. Parthenogens were more closely related to sympatric sexuals from the same population than to parthenogens elsewhere.

Here, we studied the phylogenetic relationships of sexual and parthenogenetic *S. polychroa* collected at 38 sites in Europe to explain their distribution. In a first step we review all available distribution data from the literature. Using an mtDNA marker, we then investigate (1) whether parthenogens arose repeatedly or whether they are monophyletic, and (2) whether there are indications for recent or ancient parthenogenetic strains. Special attention was paid to mixed populations and to evidence for hybridisation between sexuals and parthenogens. We discuss what these data imply for the recolonisation of Central and Northern Europe by sexual and parthenogenetic *S. polychroa*.

### ***Schmidtea polychroa*: Diploid sexuals and polyploid parthenogens**

*Schmidtea* (formerly *Dugesia*) *polychroa* consists of four karyologic forms, or *biotypes* A to D [28]. B, C and D represent polyploid, parthenogenetic forms derived from the diploid sexual biotype A ( $2x = 8$ ). Benazzi [28] differentiated between synaptic (B) and asynaptic (C, D) oogenesis. In the synaptic biotypes, production of triploid or tetraploid eggs is achieved by endoduplication of the polyploid chromosome set, followed by meiosis during which the identical, duplicated sister chromatids pair. This means that normal meiotic processes, including segregation and recombination, occur, but eggs still have the same genetic information as their mother. The other biotypes produce triploid (C) and tetraploid (D) eggs mitotically. In all parthenogens (B–D) egg development requires fusion with allosperm. The sperm nucleus, however, does not fuse with the egg nucleus, but degrades and is expelled [29].

*Schmidtea polychroa* is a generalist and can be found in meso- to eutrophic freshwater habitats like lowland rivers, streams, ditches, and lakes. Sexuals and parthenogens are widespread and live in sympatry in several localities in Italy. Sexuals have never been reported from Central and Western Europe, but are present in Sweden (see results). It is therefore unlikely that differences in climatic preferences or variance in colonization ability alone can explain the distribution pattern.

## **Results**

### **Distribution of sexual and parthenogenetic *Schmidtea polychroa***

Sampling coverage is good for much of Europe, except for Eastern Europe and the Iberian Peninsula (Table 1). Large numbers of collection sites in close proximity of a single study area [e.g. locality 30] were pooled as a single locality. Due to a large number of data points in some areas, not every single location was listed when no additional biotype was found. This applies to the British Isles and some Mediterranean islands.

**Table 1: Review of *S. polychroa* distribution data.** \* indicates that ploidy composition varies between sites within the same locality. **A** = diploid, sexual, **B** = triploid or tetraploid parthenogenetic with synaptic oogenesis, **C** = triploid parthenogenetic with asynaptic oogenesis, **D** = tetraploid parthenogenetic with asynaptic oogenesis, **x** = parthenogenesis type not identified.

Location	2x	3x	4x	Reference
<b>Mediterranean Islands</b>				
Elba, Rio Elba	A			[28]
Sardegna, locality not specified	A			[28]
Sardegna, 7 localities (streams, rivers) on the Northern part of the island	A			[68]
Sardegna	A	C	D	[69]
Sicilia, Palermo		C		[28]
<b>Italy</b>				
Napoli (Sarno)		B		[28]
Roma		C		[28]
Lago Trasimeno and surroundings	A			[26]
Lago di Chiusi	A			[26]
Perugia		C		[28]
Camerino	A			[28]
Follonica		C	D	[28]
Maremma toscana		B		[28]
Pisa and surroundings	A			[28]
Monti pisani, several sites (streams, ditches)	A	x	x	[25], [26], [45], [70]
Vaiano	x	x	x	J. Zeitlinger (unpublished data)
Forli	A			[28]
Firenze		C		[28]
Pallanza		B		[28]
Starno		B		[28]
Torino	A			[28]
Pavia		B		[28]
Ferrara	A			[28]
Lago di Mergozzo	A			[48]
Lago Maggiore*	A	B		[48]
Lago Maggiore	A			[27]
Lago Maggiore		B		[28]
Lago di Varese		x		this study
Lago di Lugano		x	x	this study
Laghi di Mezzola / Annone		B		[28]
Lago di Como,		x	x	this study
Lago di Como, Santa Marina		x		[25]
Lago di Garlate		x	x	[25], this study
Lago d'Iseo		B		[71]
Lago d'Iseo	A	B		[72]
Lago d'Iseo*	A	x	x	this study
Lago d'Idro	A			this study
Lago di Garda	A			[28], this study
Lago di Garda	A	B		[27]
Sarca / Arco	A			[28]
Sarca / Arco	A	B		this study
Lago di Toblino	A			[25], [26], [28]
Lago di Caldonazzo		B		[28]
Lago di Caldonazzo*	A	x	x	[21], [25], [26]
Lago di Levico	A			[25], [26]
Brenta / Levico	A		x	this study
Lago di Pietrarossa, pr. Monfalcone	A			[28]
<b>Austria</b>				
Graz	A			[73]
Ossiacher See		x	x	this study
Millstaedter See		x	x	this study
Fuschl-See		x	x	this study

**Table 1: Review of *S. polychroa* distribution data.** \* indicates that ploidy composition varies between sites within the same locality. **A** = diploid, sexual, **B** = triploid or tetraploid parthenogenetic with synaptic oogenesis, **C** = triploid parthenogenetic with asynaptic oogenesis, **D** = tetraploid parthenogenetic with asynaptic oogenesis, **x** = parthenogenesis type not identified. (Continued)

Donau, Wien		x		[25]
<b>Hungary</b>				
Velencei-tò	A			this study
Feher-tò	A			this study
<b>Spain</b>				
Rio Henares, Chilieches, Alcala de Henares	A			[74]
Algemesi, Valencia	A	x		[49]
Rio Palancia, Sagunto, Prov. Castelló	A			this study
<b>Germany</b>				
Main, Zellingen		x	x	[25]
Main, Hallstadt		x		[25]
Regnitz, Pettstadt		x	x	[25]
Regnitz, Hüttendorf		x	x	[25]
Altmühl, Treuchtlingen		x	x	[25]
Tübingen	B			[28]
Ammersee, Herrsching		x	x	[25], [26]
Ammersee, Wartaweil		x	x	L. Gerace, M. Storhas (unpublished data)
Maisinger See, Maising		x		[25]
Isar, München		x	x	[26]
Würm, Starnberg		x		[25]
Starnberger See, Feldafing		x		[25]
Riegsee, Murnau		x		[25]
Tegernsee		x		M. Storhas (unpublished data)
Chiemsee, Chieming		x	x	[25], [26]
Waginger See, Tettenhausen		x	x	[25], [26]
Frankfurt			B	[28]
Würzburg	B			[28]
Mainz	B			[28]
Münster		x		M. Storhas (unpublished data)
Plön, 3 lakes in the surroundings		x	x	this study
<b>Switzerland</b>				
Aarau		B		[28]
Zürichsee		B		[28]
Lago di Lugano, Riva San Vitale		B		[28], this study
Lago Maggiore, Locarno		x	x	this study
<b>France</b>				
Nizza			D	[28]
Gardon		x		[25]
Lac de Nantua		x		[25]
Montpellier		B		[71]
Tet / Perpignan		x	x	this study
Lac Lemán, Nernier		x		this study
Lac d'Annecy, Annecy		x		this study
Lorraine		x	x	[75]
Strasbourg		B		[28]
Les Rinfillières		x		this study
Paris		B		[28]
Paris, surroundings		x		[76]
<b>Luxemburg: Moselle &amp; Sure, several sites</b>				
		B		[46]
<b>Belgium: Kempens Kanaal, Turnhout</b>				
		x		Beukeboom et al. (1996)
<b>The Netherlands</b>				

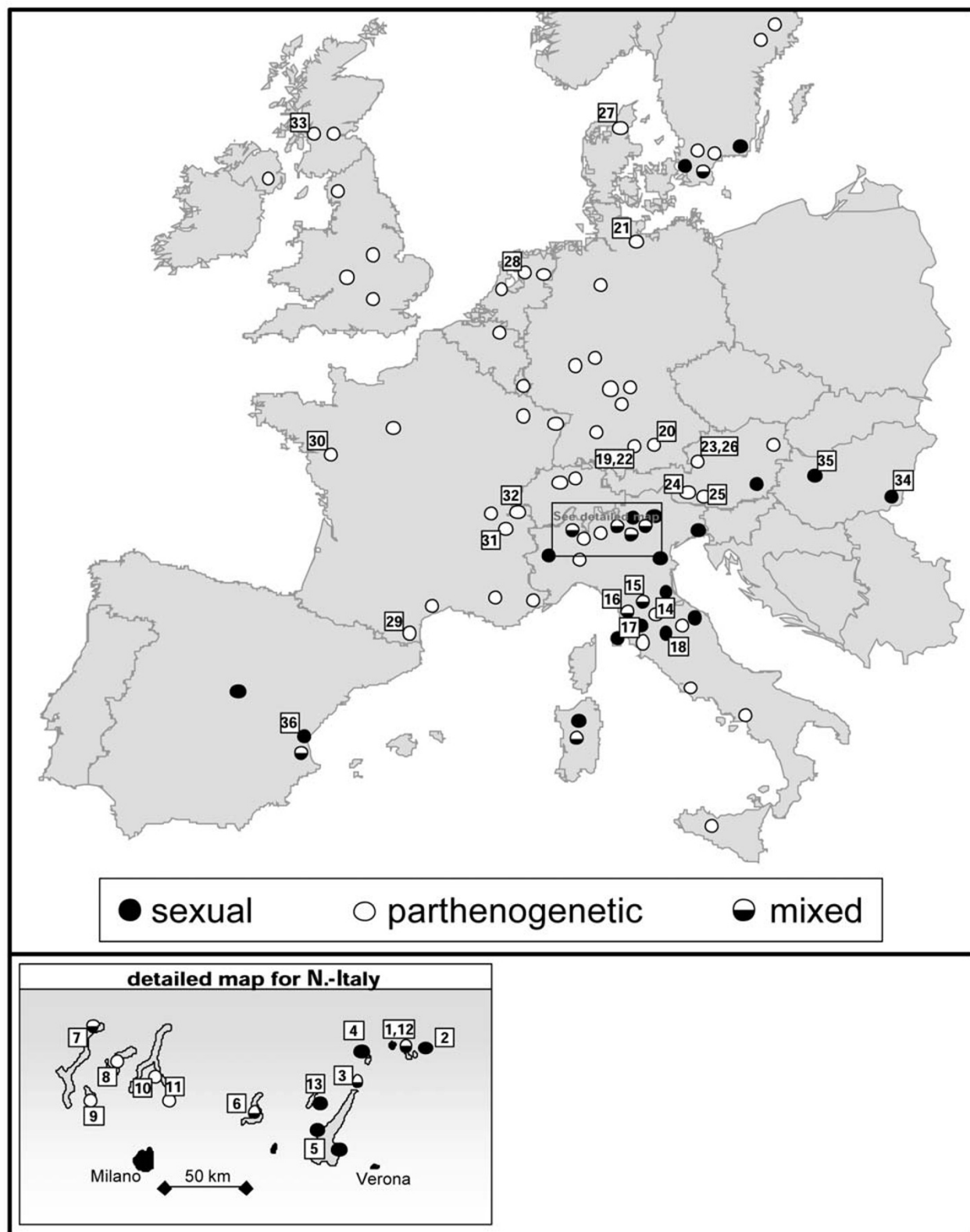
**Table 1: Review of *S. polychroa* distribution data.** \* indicates that ploidy composition varies between sites within the same locality. **A = diploid, sexual, B = triploid or tetraploid parthenogenetic with synaptic oogenesis, C = triploid parthenogenetic with asynaptic oogenesis, D = tetraploid parthenogenetic with asynaptic oogenesis, x = parthenogenesis type not identified.** (Continued)

Abcoudermeer, Abcoude	x	x	[25]
't Gein, Abcoude	x		[25]
De Vecht, Uitermeer	x	x	[25], [26]
De Vecht, Hinderdam	x	x	[25]
Spiegelplas, Hinderdam	x	x	[25]
Hilversums Kanaal, Kortenhoef	x	x	[25]
<b>Denmark</b>			
Copenhagen	B		[28]
Lake Färe	B		[28]
Kundsø	B		[27]
Ålborg, lake		x	this study
<b>Sweden</b>			
Lake Mälär	B		[28]
Lake Ivsjön	B		[27]
Värnamo			[27]
Hjälmarén	B		[27]
Lake Yddingen	A	B	[27]
Lake Vansbjön		B	[27]
The Baltic, E. of Småland	A		[27]
Lake Eketräsk	A		[27]
Scania	B		[27]
<b>Great Britain</b>			
Cole Mere, The Mere, Windermere, Johnston Loch, Loch Linlithgow	B		[77]
Sheffield	x		[26]
Oxford	B		[28]
Shropshire	B		[73]
Wales (not specified)	B		[27]
S. Andrew	B		[28]
Loch Bardowie, Glasgow	x		this study
<b>Northern Ireland</b>			
Armagh, 7 lakes in the surroundings	B, C		[78]
<b>Canada</b>			
St. Lawrence River, Cornwall (Ontario)	x		[53], [79]
Lake Ontario, Prinyer Cove, near Picton	x		[53], [80]
<b>United States of America</b>			
Lake Champlain, Burlington (Vermont)	x		[53], [79]

The majority of studies reported *either* sexuals *or* parthenogens for a given locality (Fig. 1). Sexual populations are abundant in Italy, including Sardinia and Elba. Reports from sexual forms elsewhere are rare, but span a wide geographical range: Hungary (2 sites), Austria (Graz), Spain (3 sites) and Sweden (3 sites). Sexual populations have not been found North and West of the Alps, including France, Germany, Switzerland, large parts of Austria, the Netherlands, Belgium, Denmark, Great Britain and Ireland. In all those countries, only polyploid (mainly trip-

loid) populations have been reported. No data are available for much of Eastern Europe, except for the more general statement that *Dugesia* [=Schmidtea] *polychroa* reaches its eastern distribution limit at the Volga [31,32].

Parthenogenetic *S. polychroa* are found all over Europe. Biotype B appears to be most widely distributed, but one needs to consider that most studies do not distinguish between biotypes B, C and D. Mixed populations of sexuals and parthenogens were only reported from Pisa, Italy

**Figure 1**

Overview of the geographic distribution of diploid sexual and polyploid parthenogenetic *Schmidtea polychroa* in Europe. Numbers indicate sample ID for COI phylogeny (see Table 2).

**Table 2: Newly sampled localities with karyotypic and mtDNA composition for 2x (diploid sexuals) and 3x - 4x (triploid and tetraploid parthenogens). N=number of individuals karyotyped, n=number genotyped, COI=haplotypes based on mtDNA COI sequence (Fig. 2).**

Region	No.	Locality	Sexuals (2x)		Parthenogens (3x)		Parthenogens (4x)	
			N(n)	COI	N(n)	COI	N(n)	COI
Northern Italy	1	Lago di Caldonazzo	8(8)	1, 7, 15	13(13)	1, 6, 7, 14	5(5)	6, 7
	2	Lago di Levico	7(7)	1, 22	-	-	-	-
	3	Sarca	93(27)	1, 3, 16, 29	1(1)	3	-	-
	4	Lago di Toblino	1(1)	1	-	-	-	-
	5	Lago di Garda	10(6)	3, 10, 11, 28	-	-	-	-
	6	Lago d'Iseo	91(8)	3, 14	24(5)	3, 14	3(2)	3, 14
	7	Lago Maggiore	-	-	15(4)	6, 17	5(1)	17
	8	Lago di Lugano	-	-	24(4)	3, 6, 25	3(1)	3
	9	Lago di Varese	-	-	3(3)	3	-	-
	10	Lago di Como	-	-	12(5)	3, 5, 6	3(0)	-
	11	Lago di Garlate	-	-	11(4)	6, 26	-	-
	12	Brenta	3(2)	1	-	-	1(1)	6
	13	Lago d'Idro	2(2)	3	-	-	-	-
Central Italy	14	Vorno	-	-	-	-	1(1)	19
	15	S. Lorenzo Vaccoli	2(2)	23	-	-	1(1)	19
	16	La Fattoria: ditch	1(1)	18	-	-	1(1)	19
	17	Lago di Chiusi	2(2)	2	-	-	-	-
	18	Lago Trasimeno	4(4)	2	-	-	-	-
Germany	19	Ammersee	-	-	5(5)	3, 8, 17	-	-
	20	Chiemsee	-	-	1(1)	3	-	-
	21	Plön	-	-	7(2)	20, 27	-	-
	22	Isar	-	-	1(1)	30	-	-
Austria	23	Fuschl-See	-	-	16(3)	3, 8	4(1)	3
	24	Millstaedter See	-	-	2(1)	3	2(1)	3
	25	Ossiacher See	-	-	16(4)	3, 8	1(1)	3
	26	Mondsee	-	-	1(1)	3	-	-
Denmark	27	Alborg	-	-	-	-	4(3)	24
Netherlands	28	De Vecht	-	-	2(2)	3	-	-
France	29	Têt	-	-	10(3)	3, 4	4(1)	4
	30	Nantes	-	-	13(3)	3	-	-
	31	Lac d'Annecy	-	-	8(5)	4, 21	-	-
	32	Lac Lemman	-	-	10(4)	8, 12, 13, 21	-	-
Scotland	33	Loch Bardowie	-	-	1(1)	8	-	-
Hungary	34	Feher-tò	24(7)	9	-	-	-	-
	35	Velencei-tò	1(1)	9	-	-	-	-
Spain	36	Palancia, Sagunto	4(3)	31	-	-	-	-
Romania	37	Aiud	biotype E (outgroup) biotype F (outgroup)					
	38	Huedin						

(Monti Pisani: [25,30]), North Italian lakes (Lago Maggiore: [33]; Lago di Caldonazzo: [22]; Lago d'Iseo: this study), and one locality in Spain [34].

#### Phylogenetic relationships among COI haplotype sequences

From 176 individuals we identified 31 different sequences (haplotypes hp01-31; Fig. 2 and 3). Within haplotypes, letters were used to differentiate between individuals with the same haplotype but different ploidy (2x, 3x or 4x) or geographic origin. This resulted in 82 *S. polychroa* sequences for phylogenetic analysis and allowed the comparison of single or multiple origins of parthenogens in the analysis (see below). Note that hIE03 (*S. lugubris*, biotype E) and hIF01 (*S. nova*, biotype F) were used as outgroups.

Within *S. polychroa*, polymorphisms were found at 59 positions, 28 of which were parsimony informative. Forty-nine out of a total of 63 substitutions were synonymous. Many of the rare haplotypes differed by a single substitution from a common haplotype (hp03, Fig. 2 and 3). Within *S. polychroa*, the largest genetic distance was 24 nucleotides, or 6.2% (Table 3). Between *Schmidtea* species, the differences ranged between 61 and 87 substitutions. *S. polychroa* sequences, sampling site and biotype information are accessible in GenBank (AF287052 – AF287133).

Both phylogenetic methods (Bayesian and maximum-likelihood ML) gave very similar results recovering most monophyletic groups with posterior probability  $\geq 90\%$  (Fig. 2). In both analyses parthenogens (3x and 4x) were scattered on several branches indicating repeated origin from sexuals. To test this possibility, three constraint analyses were performed using the Shimodaira-Hasegawa test (one-tailed). The first assumed a single origin for all parthenogens (both 3x and 4x). The next two assumed a single origin for either 3x or 4x parthenogens (Table 4). All three are significantly worse than the Bayesian phylogeny and therefore we reject the possibility of a single origin for parthenogenesis. Figs. 2 and 3 show that a single haplotype (hp03) is widespread among Central European parthenogens. The four sexual *S. polychroa* with hp03 are restricted to 4 lakes in Northern Italy (Fig. 2).

The network analysis shows hp03 as a central, presumably ancestral haplotype in the TCS analysis. Some haplotypes could not be joined without exceeding the maximum number of mutational steps as specified by the parsimony criterion. One of these was the very divergent hp31 from the only Spanish sexual *S. polychroa*. Another four (hp06, hp14, hp25 and hp26) clustered together in a small separate network. All haplotypes within this cluster are from Northern Italy (Fig. 3). Two other divergent groups

were represented by parthenogens from France (hp12, hp13 and hp21) and sexuals from Central Italy (hp02, hp18 and hp23). Three more highly divergent, single haplotypes (hp05, hp17 and hp19) were only found in parthenogens.

From all sampled areas, Northern Italy shows most diversity. Although part of this must be attributed to the high sampling effort in this area, it is also the region where two widespread, but distinct clusters overlap. These two differ by about 5% and consistently appeared on different branches of the tree, irrespective of the method used.

#### Parthenogenesis: recent or ancient?

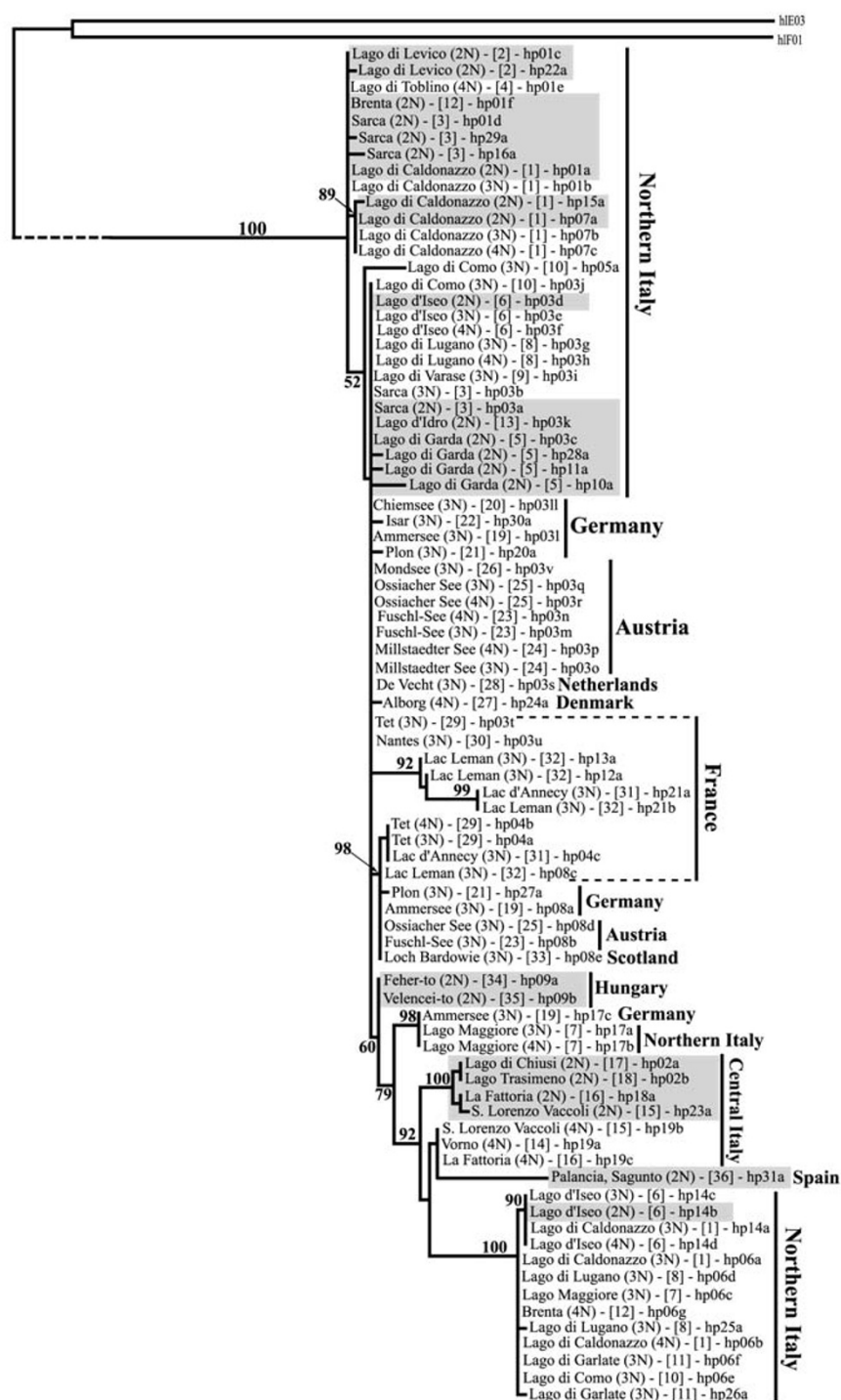
The degree to which parthenogenetic lineages diverge from their closest sexual progenitors should correlate with their ability to persist for a time long. Differences between parthenogenetic strains in the degree of divergence would then be a relative measure of the age of such lineages, and allow to distinguish relatively "young" from relatively "old" lineages. In all main clusters both sexuals and parthenogens were found (Fig. 2, 3). Some parthenogenetic lineages had haplotypes identical or similar to sympatric or geographically close sexuals, indicating recent (sexual) ancestors (Table 2, Fig. 2). Other parthenogens, however, showed large differences to nearby sexual haplotypes. The largest observed difference was 13 nucleotide substitutions and was found between parthenogenetic *S. polychroa* from Lac d'Annecy (France) and sexual individuals from Northern Italy, which represents a divergence of approximately 3%. Hp19, present in parthenogens from Central Italy, differs by six substitutions from sympatric sexuals (hp18, hp23), a 1.8% divergence. Parthenogens in Lago di Como (hp05) and Ammersee (hp17) show a similar pattern when compared to the nearest sexual populations. Divergent lineages like these may represent ancient parthenogenetic lineages. However, it cannot be excluded that some more closely related sexual haplotypes are present in the field, but were not sampled.

## Discussion

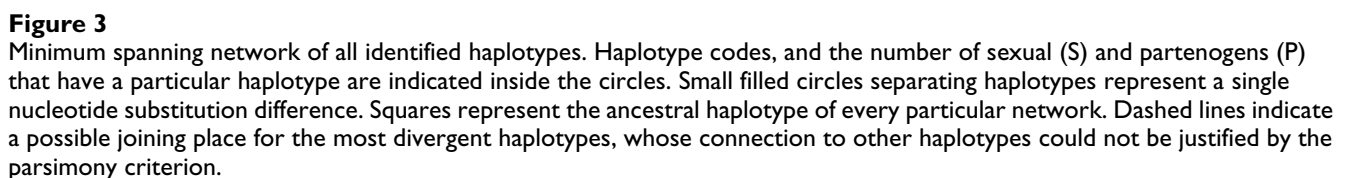
#### Geographic distribution

Although the distribution of sexual and parthenogenetic *S. polychroa* suggests 'geographic parthenogenesis' [4], the picture is complicated. On a large scale, the pattern is clear: sexuals are absent from areas North of the Alps, including France, Germany, parts of Austria, Belgium, Luxembourg, the Netherlands, Denmark, and Great Britain. Yet, mixed populations occur in Italy, Spain and Sweden. Data for Eastern Europe are poor, but sexuals occur in Hungary. Since sexuals have never been observed in any other place North of the Alps, their occurrence in Sweden raises questions about long-distance dispersal aided by waterfowl [35,36] or humans. Anthropogenic dispersal is invoked for the success of the North-American *Girardia*



**Figure 2**

Bayesian phylogenetic analysis of haplotype sequences hp01-hp31 of *S. polychroa*. hIE03 (*S. lugubris*, biotype E) and hIF01 (*S. nova*, biotype F) were used as outgroups. Numbers adjacent to the nodes indicate the posterior probability for the Bayesian analysis. Locality names are followed from left to right by ploidy number (between brackets), locality name (between square brackets) and haplotype code. Haplotypes with equal number but followed by a different letter (e.g. hp03a, hp03b, hp03c, etc.), represent identical haplotypes (identical COI sequences) found in animals with different ploidy and/or from a different geographical locality. Grey boxes highlight all sexual (diploid) *S. polychroa* included in this study. Outgroup branch with dashed line has been shortened for aesthetics of the figure.



As Scandinavia was ice-covered during the last glaciation, present-day populations in Southern Sweden must be descendants of lineages that persisted in refugia. The Baltic Sea may have aided dispersal into Scandinavia, since its salinity dropped to freshwater levels during certain peri-

ods ('*Ancylus* Sea') [39]. At present, several freshwater planarians, including *Schmidtea polychroa*, can be found in brackish regions along the Swedish East coast [27,40]. Colonization of Scandinavia after the last glaciation through the Baltic has been shown for the European perch using genetic data [41,42]. The fact that sexual *S. polychroa* are absent from Denmark and Germany suggests that Sweden was colonized by Eastern populations. Extensive karyological and genetic data from Eastern Europe and Scandinavia are needed to confirm this possibility.

**Table 3: Genetic distances between 13 most divergent haplotype sequences from a total of 31 different sequences (hp01-31). Below diagonal: nucleotide substitutions; above: raw distance in %.**

	1	2	3	5	6	8	9	10	12	17	19	21	31
1	-	2.08	0.78	1.82	4.16	1.04	1.04	1.82	2.08	2.34	2.34	3.12	4.94
2	8	-	2.86	3.90	3.38	3.12	2.60	3.90	3.12	2.86	1.82	2.08	4.68
3	3	11	-	1.56	4.16	0.26	0.26	1.04	1.82	1.56	2.08	3.38	4.68
5	7	15	6	-	5.71	1.82	1.82	2.60	3.38	3.12	3.64	4.94	6.23
6	16	13	16	22	-	4.42	3.90	5.19	4.42	4.16	2.86	3.90	5.45
8	4	12	1	7	17	-	0.52	1.30	2.08	1.82	2.34	3.64	4.94
9	4	10	1	7	15	2	-	1.30	2.08	1.30	1.82	3.64	4.42
10	7	15	4	10	20	5	5	-	2.86	2.60	3.12	4.42	5.71
12	8	12	7	13	17	8	8	11	-	2.86	2.86	1.56	5.97
17	9	11	6	12	16	7	5	10	11	-	2.08	3.90	4.68
19	9	7	8	14	11	9	7	12	11	8	-	2.86	3.38
21	12	8	13	19	15	14	14	17	6	15	11	-	5.71
31	19	18	18	24	21	19	17	22	23	18	13	22	-

**Table 4: Statistical support for alternative hypotheses on Schmidtea phylogenetic relationships. (SH, Shimodaira-Hasegawa test: Comparison between the constrained and unconstrained (first, Bayesian) solution)**

Tree	- Log likelihood	$\Delta$ -Log likelihood	p (SH)
Unconstrained Bayesian tree (Fig.2)	1432.3	(best)	
Single origin for all parthenogens (3x + 4x)	1525.1	92.8	< 0.001
Single origin for all triploid <i>S. polychroa</i>	1550.0	117.7	< 0.001
Single origin for all tetraploid <i>S. polychroa</i>	1556.3	124.0	< 0.001

Our (limited) genetic data for parthenogens from Denmark suggests that they are descendants of Central European populations. It suggests that the Kattegat Strait between Denmark and Sweden is a dispersal barrier for *S. polychroa*. Support for this possibility comes from another planarian, *Polycelis nigra*, which occurs throughout Europe up to Northern Germany and Denmark, but is missing from Sweden [43]. Yet, *P. nigra* is one of the commonest European planarians and often lives under conditions similar to those prevalent in southern Sweden [35].

#### Phylogeography of European species, and geographic distribution of haplotypes

Hewitt [44] analysed the effects of recurrent ice ages and concluded that species ranges have contracted and expanded repeatedly. The consequences are loss of variation, divergence among populations due to isolation, genome reorganization and hybridisation of slightly divergent genomes [44,45]. In principle, geographic and genetic variability allow identification of recolonisation routes and hybrid zones after the last ice ages [10,11]. However, in our data the intraspecific divergence among mtDNA lineages is so extreme, that it appears as if major lineages split long before the quaternary ice ages. This

means that the latter did not cause, but possibly maintained or reinforced existing divergence [11].

During the ice ages not all species went extinct in Central Europe. Cold-adapted species survived in the ice-free corridor between the Scandinavian and the Alpine ice sheets. Some planarians, including *S. polychroa*, may well have belonged to this community [46]. Planarians, including *S. polychroa*, also survived the last ice age in Britain's ice-free Southwest [35]. This implies that recolonization of previously glaciated habitats may well have started from persisting nearby populations rather than from remote refugia. Although low temperatures in ice-covered lakes does not seem to affect adult survival, *S. polychroa* requires temperatures to rise above 7.5°C for cocoon production [35]. This coincides with Central European summer temperatures during glaciations, which were around 9°C [47].

With few exceptions, clusters of related haplotypes belong to certain geographical regions (Fig. 2). Spanish samples differed clearly from all others. Similarly, no haplotypes were shared between Central Italy and any other region. Two very different clusters were exclusively found in

Northern Italy, coexisting with even more types. North of the Alps, however, haplotype variation is low. This does not necessarily indicate recolonization from a single source population after the last glaciation. The COI gene may be too conserved to allow resolution of divergence caused by the most recent glaciation. In contrast to other phylogenetic studies, the Alps do not seem to represent an absolute genetic barrier for some haplotypes. Hp03, dominant in Central Europe parthenogens, also occurs in Northern Italy. However, the Alps do seem to represent a dispersal barrier for sexual, diploid *S. polychroa*, which are absent from countries on their western and northern edge (France to Austria).

#### **Origin and divergence time of parthenogenetic lineages**

Our results support the multiple, repeated origin of parthenogenetic lineages [26] from sexuals. A single origin is rejected by the constraint analyses (Table 4).

Inferring the age of a parthenogenetic lineage from genetic data is based on nucleotide divergence with its closest sexual relative (the hypothetical ancestor). If the true ancestor has gone extinct or has not been sampled, this procedure overestimates the age of the lineage. It also requires a reliable molecular clock for the focal sequence. Nevertheless, if the sampling pattern is dense enough, at least a qualitative identification of "ancient" parthenogens should be possible. Estimates for the mutation rate of the mitochondrial COI gene of arthropods are in the range of about 2% per million years [48–50]. Although mutation rates differ between taxa, we apply this rate to *S. polychroa* for a preliminary estimate. It suggests that most parthenogens are not older than 500,000 years. The parthenogenetic lineages from France (hp13, hp12 and hp21), however, differ by about 1.5 to 3% from their closest sexuals. This suggests divergence times of 750,000 to 1.5 million years. If this is true, parthenogens from the same or adjacent areas should form a monophyletic clade of exclusively and rather ancient parthenogenetic lineages. Better phylogeographic coverage for Southern France and Switzerland is required to prove this.

Finally, tetraploid parthenogens of localities 14 and 15 (Fig 1) differ strikingly from coexisting sexuals (Fig 2 and 3). Further sampling from more populations from adjacent areas is required to confirm their relationship. As the Italian distribution of sexuals and parthenogens is a true mosaic, these tetraploids may be descendants from extant but as yet unsampled diploid populations.

#### **Hybridisation between sexuals and parthenogens in mixed populations**

Mixed populations encourage ecological and genetic interactions between sexuals and parthenogens, allowing the study of the evolutionary advantage of sex and the ori-

gin of parthenogenesis. Hermaphroditic parthenogens that have a functional male gender may spread genes for parthenogenesis in the sexual gene pool, thus diluting and ultimately displacing sexuality. Jaenike & Selander [51] explored the ecological conditions under which such processes work and applied them to explain the distribution of parthenogenetic oligochaetes in North America.

Being a hermaphrodite, *S. polychroa* always has both sex functions in the same individual, also in parthenogens. Unusual is that parthenogens are sperm-dependent and produce haploid sperm, an unusual trait for a polyploid [53]. They require sperm from a partner (sexual or parthenogenetic) to activate parthenogenetic development of their eggs [52]. However, neither sexuals nor parthenogens are able to self-fertilize. Hybridisation between sexuals and parthenogens has been studied extensively in the population of Lago di Caldonazzo (Trento, N-Italy). The frequency distribution of sexuals and parthenogens varied strongly between sites but was not explained by ecological parameters [22]. Genetic and karyological data indicate that new triploid, parthenogenetic lineages arise regularly as a result of hybridisation between sexuals and parthenogens [21,26,54]. Higher heterozygosity and diversity among parthenogens may be attributed to triploidy and fixed heterozygosity. However, it is also evidence for secondary contact of sexuals and parthenogens. If parthenogens had exclusively originated from local sexuals, their genetic variability should be within that of sexuals, which is not the case. Our results show that although some haplotypes are shared (Hp01, Hp07), others are exclusive to parthenogens (Hp06, Hp14). This pattern is supported by a detailed study with large samples from several sites within Caldonazzo [54]. Interestingly, parthenogenetic lineages differ not only with regard to their mitochondrial COI sequence, but also with regard to sex allocation and female fecundity. This indicates that they may represent different stages of adaptation to clonal reproduction [54].

#### **Conclusions**

Sexual *S. polychroa* are absent from Central and Western Europe, where populations are exclusively parthenogenetic. The pattern South of the Alps reveals a complicated overlapping mosaic between parthenogens and sexuals. Coexistence also exists in Spain and Southern Sweden. Clustering of COI haplotypes identified groups of geographically close lineages. In northern Italy, two haplotype clusters are present that differ by about 5%, but overlap geographically. Both types were found among sexuals as well as parthenogens. The data suggest that postglacial colonisation in Europe was not from a single refugium, but from several sources that already had diverged to some extent. It is likely that Scandinavia was colonised from the East, whereas Central and Western Europe were colonised from the South or Centre. The data

further indicate that parthenogenetic lineages are present on several branches in a phylogeographic tree, indicating repeated origin. The latter may be enhanced by hybridisation between sexuals and parthenogens, as shown in studies using microsatellites [54]. This can explain the coexistence of identical sexual and parthenogenetic haplotypes. Yet other parthenogenetic haplotypes differed clearly from coexisting sexuals, indicating dispersal and secondary contacts. Coexistence of sexual and parthenogenetic forms must therefore be seen as a dynamic process of genetic exchange, arisal (and extinction) of new lineages, and dispersal. Exclusive parthenogenesis in Central and Western populations suggests better colonisation capacity of parthenogens. It is unclear why this is, but since sexuals are obligate outcrossers, they may suffer from inbreeding costs in small founder populations, slowing down their colonisation rate relative to parthenogens. Sexuals may also be poor competitors when invading already established parthenogenetic populations. Sexuals cannot obtain paternity in parthenogenetic partners, and even "help" to generate new parthenogenetic lineages through hybridisation.

## Methods

### Review of distribution data

Distribution records were extracted from publications and personal communications. All studies with karyological data and a description of sample origins were considered, but with an emphasis on continental Europe. Because field samples were collected differently in different studies, only presence or absence of a certain ploidy level was scored, not abundance. Differentiation of biotypes B, C and D requires cytological analysis of the final stages of oogenesis in fresh cocoons. Most studies, however, were limited to simple chromosome counts and only allow identification of (diploid) sexual and (polyploid) parthenogenetic lineages (Table 1).

### Own data

Additional data were obtained from field trips to 38 localities in Europe between 1996 and 1999. Collection, transportation, and preparation of metaphase chromosome spreads are described in Pongratz [26]. For two samples (Sarca and Palancia), ploidy was not determined by karyotyping but inferred from allele numbers at up to four, highly polymorphic microsatellite loci (data not shown) [55].

### mtDNA sequencing

Genomic DNA was extracted following the protocol in Pongratz [55]. In total, we analysed 176 individual *S. polychroa* from 36 different localities (Table 2). 81 samples were from diploids, 75 from triploids, and 20 from tetraploids. For use as an outgroup in phylogenetic analyses, several specimens of *Schmidtea* biotype E and F [28,56]

were sequenced. These were collected in populations 37 and 38 (Table 2).

### Sequencing of COI

We amplified a fragment of the mitochondrial cytochrome-oxidase I (COI) gene using primers pr-a2 (5'-AGCTGCAGTTTTGGTTTTTGGACATCCTGAGGT-3') and pr-b2 (5'-ATGAGCAACAACATAATAAGTATCATG-3') [57]. Although these primers were developed for *Dugesia japonica*, they amplify the same region in *S. polychroa*. For details see Pongratz [58]. PCR products were purified using GeneClean® Kit (Bio 101), yielding 15 µl solution of clean template in appropriate concentration for sequencing reactions with the ABI PRISM™ Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems). Fragments were analysed on an ABI PRISM310. All samples were sequenced in both directions.

### Phylogenetic analyses

All sequences were identical in length (385 bp) and were aligned manually. Polymorphisms were analysed with DNASP3.0 [59] and DAMBE3.7 [60]. The latter was also used for translation into amino acid sequences using the flatworm mitochondrial code in order to differentiate between synonymous and non-synonymous substitutions.

A total of 82 *S. polychroa* sequences, plus one sequence from each of the two outgroup taxa, *S. lugubris* (biotype E; GenBank accession No. AF290021) and *S. nova* (biotype F; AF290023) were used in the phylogenetic analyses. Phylogenetic relationships were estimated using Bayesian analysis and maximum-likelihood (ML). Modeltest v. 3.06 [61] was used to select the most appropriate model of sequence evolution under the Akaike Information Criterion. This was the General Time Reversible model (GTR) taking into account the shape of the gamma distribution (G). ML analyses were performed in PAUP\*4.0b10 [62]. These consisted of heuristic searches involving tree bisection and reconnection (TBR) branch swapping. Bayesian phylogenetic analyses [63,64] were performed with MRBAYES v. 2.01 [65] using the GTR+G model with parameters estimated as part of the analysis and four incrementally heated Markov chains with the default heating values. The analysis ran for  $1.5 \times 10^6$  generations, with sampling at intervals of 100 generations that produced 15000 sampled trees. After the run, the log-likelihood values of sample points were plotted against the generation time and all trees prior to reaching stationarity were discarded. A 50% majority rule consensus tree was generated combining only the last 5000 sampled trees. The frequency of any particular clade of the consensus tree represent the posterior probability of that clade [65]; only values above 95% were considered to indicate that nodes were significantly supported. Intra-specific mtDNA haplo-

type networks were reconstructed using the program TCS v1.13 [66]. Where appropriate, topological constraints were generated using MacClade v.4.0 [67] and compared to our optimal topology using the Shimodaira-Hasegawa (SH) test implemented in PAUP\*4.0b10.

### Authors' contributions

NP, MS and NKM planned the study. NP carried out most field samples and did the molecular genetic analyses, a first analysis of the genetic data and drafted the manuscript for his Ph.D. MS assisted in field sampling and analysis. SC assisted in establishment of COI mtDNA markers in our system, redid the phylogenetic analyses and made the graphs. NKM supervised the work and edited the manuscript for publication. All authors read and approved the final manuscript.

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